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Expression of Osteocalcin and Transglutaminase and Labelling of Bromodeoxyuridine during Fracture Healing in the Rat Tibia†

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= Abstract = **The expression of osteocalcin and transglutaminase C(TGase C) during fracture healing was investigated with immunohistochemical studies. A transverse osteotomy was made at the proximal tibia in Sprague-Dawley male rats and immobilized with a small external skeletal fixator. The animals were sacrificed serially 1, 3, 5, 7, 14, 42 days respectively after fracture. Longitudinal sections of the healing bone were stained with polyclonal antibody against osteocalcin and TGase C, and monoclonal antibody against bromodeoxyuridine. During the intramembranous bone formation at the periosteum around the fracture site, osteocalcin was strongly expressed in the proliferating osteoprogenitor cells from the 1st day of fracture, and then, in osteoblasts, osteoid matrix and osteocytes. The expression of TGase C was weakly positive in both osteoprogenitor cells and osteoblasts. At the site of endochondral bone formation, which was first revealed 5 days after fracture, cell proliferation occurred at the periphery of cartilaginous callus where the number of cells stained with BrdU was highest. During the maturation of callus, those cells were entrapped in the chondroid matrix and became larger and larger. Osteocalcin was demonstrated in the cytoplasm of chondrocytes, while chondroid matrix was negatively stained. TGase C was found in the cytoplasm of more centrally located and matured chondrocytes as compared with osteocalcin. Osteoid matrix was stained with osteocalcin but not with TGase C. These finding may suggest that osteocalcin participates in the early phase of endochondral bone formation, while TGase C participates in the late phase, suggesting the role of TGase C in matrix stabilization. But the reason for the difference in the expression of TGase C between the endochondral bone formation and intramembranous bone formation should be further investigated. Healing of well immobilized fracture in this study was predominantly induced by intramembranous ossification rather than endochondral ossification. Periosteal osteoprogenitor cells appeared to initiate and to lead bone formation after osteotomy. These findings indicate that preservation of the periosteum is essential to achieve successful fracture healing.**

Key Words: *Transglutaminase, Osteocalcin, Bromodeoxyuridine, Fracture healing*

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INTRODUCTION

Osteocalcin is a calcium binding protein which comprises 10-20% of non-collagenous organic material of the extracellular bone matrix(Hauschka *et al.* 1975). The molecule consists 47-50 amino acid residues, with a molecular weight varying from 5200-5900, depending on the species(Hauschka 1986). One of its characteristics is the presence of two or three gamma-carboxyglutamic acid residues, for which reason it has also been called bone Gla protein. Among the many functions revealed by *in vitro* studies, the specific binding with calcium and high affinity for the hydroxyapatite crystal surface seem to be most important.

The immunohistochemical distribution analysis of osteocalcin showed its presence in osteocytes and lamellar bone matrix(Vermenlen *et al.* 1989). Therefore, osteocalcin is a bone-specific marker protein of the mature osteoblast(Lian *et al.* 1989). In this study, we used osteocalcin as a differentiation marker of osteoprogenitor cells toward osteoblast.

Transglutaminases(TGase) are a group of enzymes which cross-link the protein molecules through covalent isopeptide bond between a glutamine and lysine residue of the same or different proteins. The polyamines such as purtrescine, spermidine and spermine often act as bridges between protein molecules(Folk 1980). The enzyme has been studied in tissue involving tight structure formation such as epidermis or blood clot. During bone formation, tight adhesion of osteoid matrix might occur prior to calcification. Participation of TGase has been suggested in this step because several extracellular matrix proteins such as osteonectin, osteopontin, fibronectin and osteocalcin have been supposed to be possible substrates of TGase(Barsigian *et al.* 1991; Prince *et al.* 1991). In this experiment we tried to monitor the expression of TGase in fracture healing by immunohistochemical analysis.

The kinetics of the cells were monitored simultaneously with *in vivo* BrdU labelling of S phase nuclei. Bromodeoxyuridine(BrdU) is a thymidine analog, which can be inserted in the nuclear DNA during the S phase, used as a marker for proliferation of the cells. Therefore in the present experiment, we tried to investigate relationship among proliferation of the cells, production of extracellular bone matrix and the structural reforming process by monitorintg BrdU, osteocalcin and transglutaminase immunohistochemically.

MATERIALS AND METHODS

Animals and reagents

The adult male Sprague-Dawley rats were obtained from the Seoul National University College of Medicine Animal Sector. The animals were accommodated for two weeks and were operated on when body weight was about 250 g. All animals were maintained under an alternating cycle of light and darkness for 12 hours each, and at temperature of 20°C.

The respective type specific polyclonal antibody to TGase C was prepared as described earlier(Kim *et al.* 1990). The synthesized polypeptide sequence "IGFQDAYKRIYGTT", a partial fragment of osteocalcin, was used for antibody preparation. The polypeptide was conjugated with Keyhole lympet hemocyanin and three heads of rabbits were immunized with 0.1 mg conjugated polypeptide three times at 2 weeks intervals as a mixture with the same volume of complete Freund's adjuvant. The booster injection was done twice at 10 days intervals with incomplete Freund's adjuvant. The serum was collected and specificity was confirmed by Western blot using bovine serum albumin-conjugated synthetic peptide. BrdU specific antibody was purchased from the commercial source(Synbio).

Induction of fracture

General anesthesia was done by intraperitonal administration of pentothal sodium. The left leg was shaved and sterile draping was performed with 10% betadine solution. A small external skeletal fixator was applied to

the medial side of left tibia with two pairs of 1 mm diameter Kirshner wires. A short skin incision was made between the 2nd and 3rd wire and the tibia was exposed subperiosteally. With an electric microsaw, corticotomy was performed 10-15 mm distal to the articular surface of the proximal tibia, which was just the middle point of the two pairs of wires. A complete fracture was made at the corticotomy site by manipulation and another external skeletal fixators was applied to the lateral side of the tibia. The external fixators were tightened and the fracture site was compressed.

Preparation of the specimen

The animals were sacrificed at 1, 3, 5, 7, 14, 42 days after the operation. Two hours before sacrifice the animals were given bromodeoxyuridine 25 mg/kg intraperitoneally to label the S phase nuclei. The animals were sarcificed by cervical dislocation and the left tibia was dissected after detaching the external skeletal fixator. The Kirshner wires were pulled out carefully and the dissected bones were fixed in Carnoy's solution for 48-72 hours.

The specimens were transfered to the 10% nitric acid solution to decalcify the minerals for 8-10 hours. After completion of decalcification, the tibia was divided along the coronal plane. Each piece was processed for routine histologic preparation including dehydration with ethanol, clearing with xylene, impregnation and embedding with paraffin, and microtoming into 4 μ m thickness.

Four serial sections were obtained from the paraffin block and one of them was stained by routine hematoxylin-eosin stain. The remaining three were used for immunohistochemical staining against TGase C, osteocalcin and bromodeoxyuridine, respectively.

Immunohistochemical study

For the demonstration of TGase C, the hydrated slides were incubated with normal goat serum, followed by 1:800 diluted rabbit anti-TGase C antibody (Park *et al.* 1991). The slides were subsequently treated by biotinylated anti-rabbit IgG (Vector), avidin-biotin-peroxidase complex (Vector) and diaminobenzidine (Sigma) with hydrogen peroxide. Nuclear counterstaining was done by Harris hematoxylin and permanent mounting was done. Demonstration of osteocalcin was done using rabbit anti-osteocalcin antibody (1:800) by the same method. The confirming process for a anti-osteocalcin antibody was carried out satisfactorily by Western blot analysis and antigen-block experiment for immunohistochemical analysis (Data not shown).

Detection of S-phase cells was performed using mouse anti-bromodeoxyuridine monoclonal antibody (Synbio 1:250) and biotinylated anti-mouse IgG (Vector) after denaturation of DNA with 1 N HCl.

RESULTS

Periosteal osteoprogenitor cells in the cambium layer of the periosteum, which were positively stained with osteocalcin, proliferated especially near the fracture site. The proliferating cells could be confirmed by bromodeoxyuridine-positive nuclear staining. They actively proliferated from the 1st day after fracture, but the activity of proliferation gradually decreased thereafter(Fig. 1). Those proliferating osteoprogenitor cells had been differentiated into osteoblast and produced osteoid matrix rimmed by osteoblast(Fig. 3). The periosteal proliferation continued until 6 weeks after fracture.

The expression of osteocalcin was noted in the periosteal osteoprogenitor cells from the 1st day after fracture(Fig. 1-C) and gradually decreased thereafter. As the intercellular matrix was produced and matured, osteocalcin was observed in the matrix and its expression increased gradually. On the 5th day, osteoid matrix began to be formed and the expression of osteocalcin was noted in the matrix(Fig. 2). At the end of 1st week, the expression of osteocalcin in the osteoid matrix was stronger than that of the 5th day, but its expression in the cytoplasm of osteoprogenitor cell and osteoblast was weaker than that of the 5th day

Table 1. Expression of bromodeoxyuridine, osteocalcin and transglutaminase C at periosteal callus

Period	1 day			3 days			5 days			7 days			2 weeks			6 weeks		
	BrdU	OTC	TGC	BrdU	OTC	TGC	BrdU	OTC	TGC	BrdU	OTC	TGC	BrdU	OTC	TGC	BrdU	OTC	TGC
Periosteal osteoprogenitor cell	+++++	+		++	++	+	++	++	+	+	+	−	±	−	−	±	−	−
Osteoblast				−	++	+	−	++	+	−	+	+	−	+	+	−	+	−
Osteoid matrix				−	±	−	−	+	−	−	++	−	−	++	−	−	++	−

+++ : very strong, ++ : strong, + : weak, ± : very weak, − : negative

BrdU : Bromodeoxyuridine, OTC : Osteocalcin, TGC : Transglutaminase C

Table 2. Expression of bromodeoxyuridine, osteocalcin and transglutaminase C at cartilaginous callus

Period	5 days			7 days		
	BrdU	OTC	TGC	BrdU	OTC	TGC
Proliferating immature chondroblast	++	−	−	+	−	−
Undifferentiated chondrocyte	−	++	−	−	++	−
Matured chondrocyte	−	++	++	−	++	++
Chondroid matrix	−	−	−	−	−	−
Osteocyte				−	+	−
Osteoid matrix				−	−	++

++ : strong, + : weak, − : negative

BrdU : Bromodeoxyuridine, OTC : Osteocalcin, TGC : Transglutaminase C

(Fig. 2-C, 3-C). At the end of 2nd week, the expression of osteocalcin was noted in the osteoblast and the surrounding osteoid matrix. The osteoid matrix at a distance from osteoblasts was very weakly stained (Fig. 4).

The expression of TGase C was weakly positive in osteocalcin-positive osteoprogenitor cells from the 1st day after fracture(Fig. 1-D) and continued weakly until the 5th day(Fig. 2-D). TGase C was noted in osteoblast weakly from the 3rd day after fracture tq the 2nd week(Fig. 3-D, 4-D, Table 1).

The cartilaginous callus was first noted on the 5th day after fracture. Cell proliferation, which could be confirmed by nuclear staining of BrdU, occurred at the periphery of the cartilaginous callus(Fig. 5-B). As the cartilage island grew and matured, the proliferating immature chondroblasts were entrapped in the chondroid matrix becoming more centrally located and their cytoplasm became larger and larger. The expression of osteocalcin was noted at the cytoplasm of the differentiated chondrocyte and was not noted at the chondroid matrix(Fig. 5-C). TGase C was found at the cytoplasm of the more centrally located and matured chondrocytes than that of osteocalcin(Fig. 5-D).

1 week after fracture the chondroid matrix became calcified and transformed into osteoid matrix. The osteoid matrix showed positive reaction to osteocalcin and entrapped cells showed weak staining(Fig. 5-C). After 4 weeks, the osteoid filled in the medullary cavity, and they became trabecular bone and the activity of cell proliferation was minimal(Table 2).

LEGEONDS FOR FIGURES

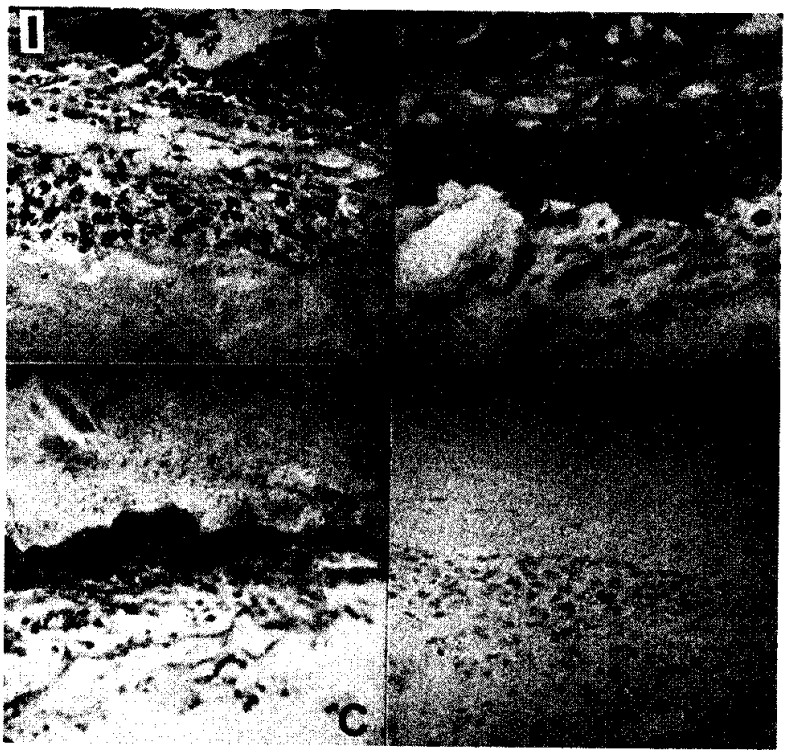
Fig. 1. Intramembranous ossification on 1 day after fracture. (A) H & E. Expression of BrdU (B), osteocalcin (C) and TGase C (D) in proliferating periosteal osteoprogenitor cells. (X100)

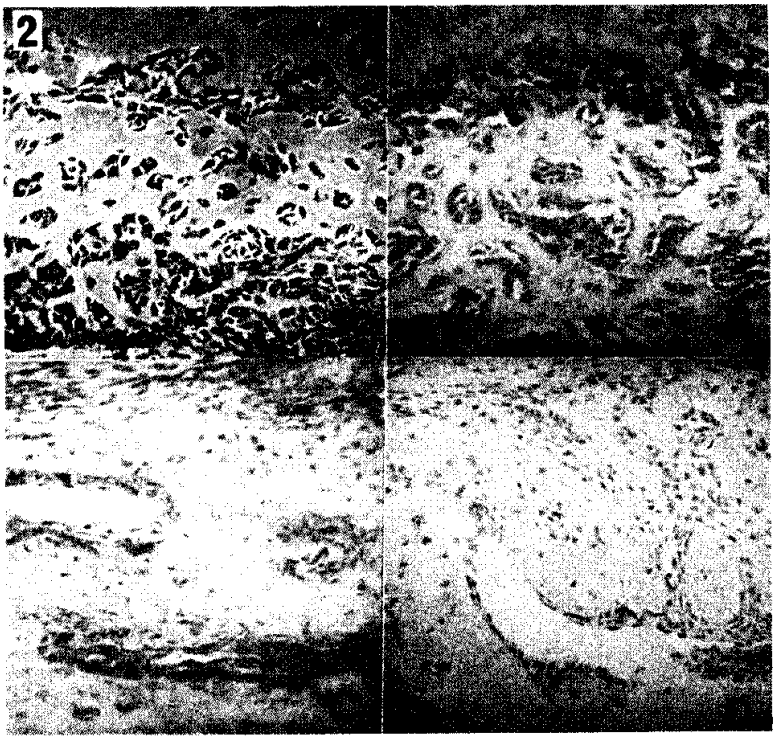
Fig. 2. Intramembranous ossification at 5 days after fracture. (A) H & E. (B) Expression of BrdU in proliferating periosteal cells. Expression of osteocalcin (C) and TGase C (D) in periosteal cells, osteoblast and osteoid matrix. (X100)

Fig. 3. Intramembranous ossification at 7 days after fracture. (A) H & E. (B) Expression of BrdU in proliferating periosteal cells. (C) Osteocalcin in periosteal cells, osteoblast and osteoid matrix. (D) TGase C in periosteal cell and osteoblast. (X100)

Fig. 4. Intramembranous ossification at 2 weeks after fracture. (A) H & E. (B) Expression of BrdU in proliferating periosteal cells. (C) Osteocalcin in osteoblast and osteoid matrix. (D) TGase C in osteoblast. (X100)

Fig. 5. Enchondral ossification at 7 days after fracture. (A) H & E. (B) Expression of BrdU at the periphery of chondroid callus. (C) Osteocalcin in chondrocyte, osteoid matrix and osteocyte. (D) TGase C-positive hypertrophic chondrocyte in the central region of the callus. (X100)

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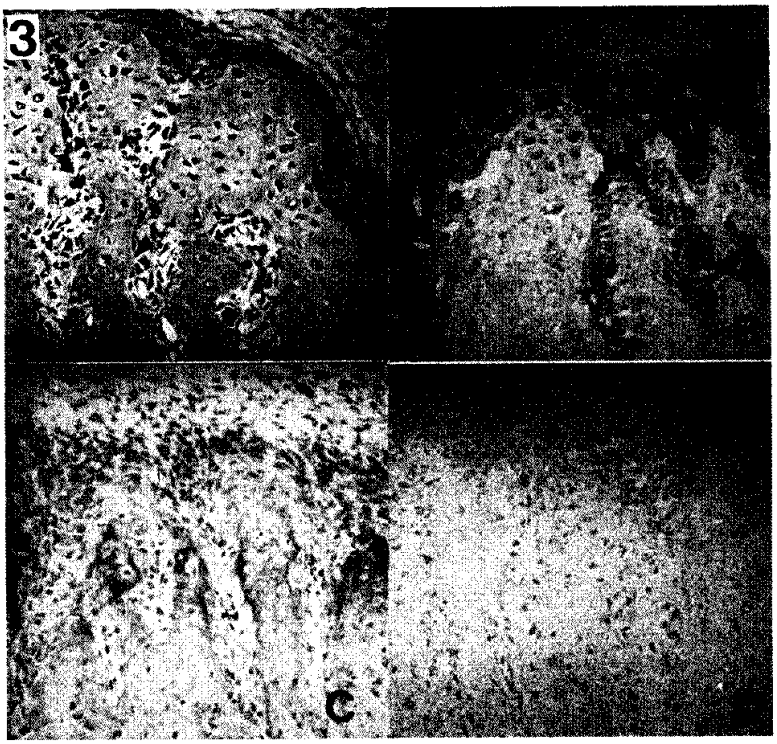
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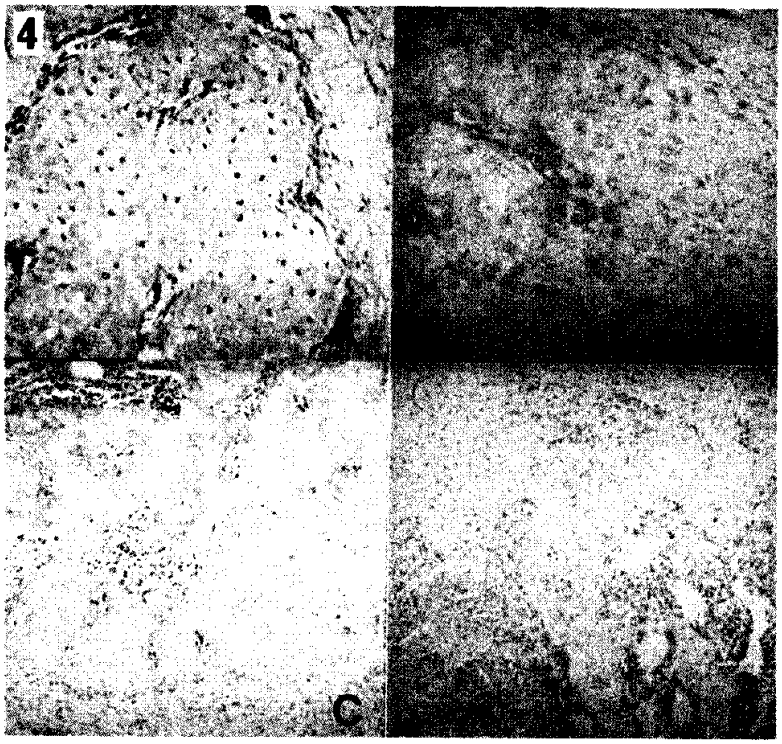
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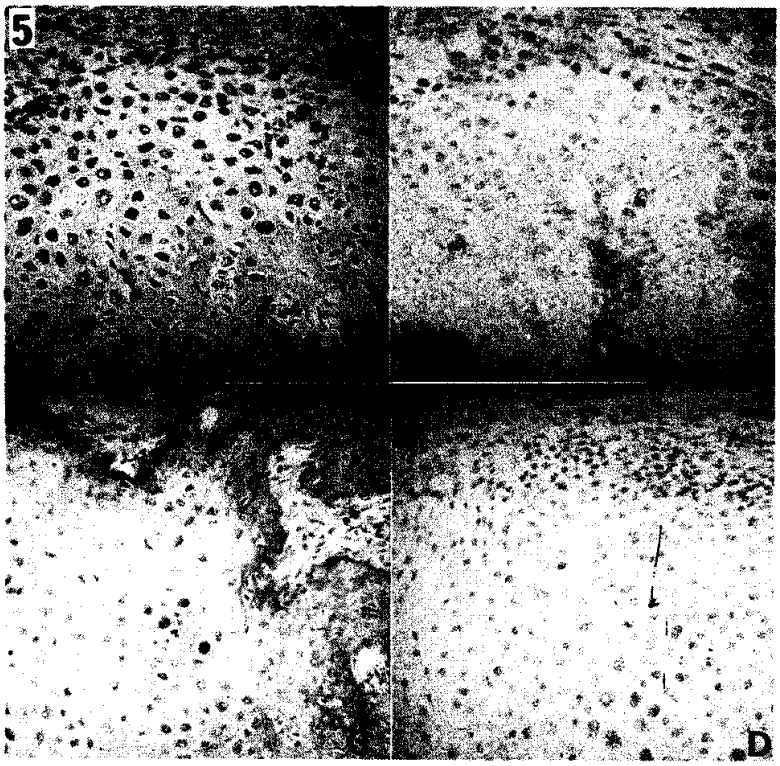
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DISCUSSION

Bone is a unique organ of the body, which completely regenerates after injury. Although liver is known for similar regeneration activity, hepatic injury often results in replacement rather than regeneration. The reason for such an excellent regeneration in bone repair is unknown, but complex growth factors and varying mitogens are known to be involved in the process of repair and differentiation of bone.

It is really surprising that few purely histologic studies on fracture repair have been published(Hulth 1988). Most of the studies have been performed with a special purpose, such as demonstrating vascularity or collagen type. McKibbin(1978) has described a model of a callus in fracture healing consisting of cells derived from two different origins. One is the primary callus response which is the proliferation of committed cells in periosteum and bone marrow. These cells directly produce membranous bone in the periosteum. The other origin is the inductive or external callus derived from the surrounding tissues. This callus is claimed to be formed by the pleuripotent mesenchymal cells with chondrogenic potential. In this experiment, intramembranous ossification predominated the endochondral ossification. This might be explained by several factors. It is already known that immobilization and right oxygen tension promote intramembranous ossification rather than chondroid formation(Von der Mark and Conrad 1979). It is known that the amount of cartilaginous callus is greater in lower animals than in higher animals(McKibbin 1978), and that the size of callus depends on the degree of movement that occurs between the fragments (Lindholm *et al.* 1969). The movement between fragments seems to start a process that gives the surrounding muscle and vessels the ability to produce mesenchymal cells and new formation of active vessels with special function. In this experiment, we applied an external skeletal fixator resulting in rigid fixation. Consequently callus formation was minimal at the fracture gap.

Immunohistochemical localization of osteocalcin has been studied by several authors using calves(Biancop *et al.* 1985), developing rat bone(Bronkers *et al.* 1985;Groot *et al.* 1986) and developing teeth in rat(Gorter *et al.* 1987). According to Vermenlen *et al.* (1989), osteocalcin immunoreactivity using polyclonal antibody is demonstrated in the osteoblast and osteocytes, where the intensity of immunoreactivity is proportional to the site of those cells. They also observed a positive reaction along the lamellar collagen in the bone matrix. Although they did not describe the immunoreactivity of chondrocytes during the callus formation, the staining characteristics seemed to be identical to our results.

According to Vermenlen *et al.* (1989), osteocalcin immunoreactivity is less preserved by strong acid solution than by a mild chelating decalcifier such as EDTA. They explained this result by the fact that during the decalcifying process, the amount of extracted osteocalcin is proportional to the duration of decalcification rather than the type of agent. Although osteocalcin is highly soluble in aqueous solution, and formalin fixation is not sufficient to fix osteocalcin completely, we could successfully demonstrate osteocalcin with processed bone specimens fixed in Carnoy solution.

In the immunohistochemical staining for osteocalcin, periosteal cells as well as some of the cartilage cells were positively stained. The osteoid matrix was also stained but chondroid matrix was negative(Fig. 1,2,3). This staining characteristic is similar to that of Stafford *et al.* (1992), who used rat polyclonal antibody. We used the rabbit antibody to synthetic partial polypeptide sequence of osteocalcin. We confirmed the specificity of our antibody by successful blocking by excess amount of antigen (data not shown). We used the antibody at the titer of 1:800 dilution and non-specific reaction was negligible.

We observed that the cartilage islands grew at the peripheral portion and they were gradually differentiated into osteoblast(Fig. 5).

The former was confirmed by BrdU labelling and the latter was suggested by the emergence of osteocalcin positivity in the entrapped cells. Our observation favors the opinion of Stafford *et al.* (1992), who insisted that the surrounding soft tissue did not contribute to the fracture callus as opposed to the concept of McLean and Urist(1968) or McKibbin(1978). In this experiment, it is evident that the osteocalcin-positive osteoprogenitor cells at the vicinity of the cortex are derived from the periosteal cells rather than the surrouding soft tissue. During the 1st day, the proliferating cells are concentrated at the periosteum and the soft tissue mesenchymal cells do not reveal such an active proliferation during the fracture healing (Fig. 1). The proliferating cells are strongly positive to osteocalcin from the beginning and they form the osteoid matrix. which is also osteocalcin positive after 5 days(Fig. 2, 3, 4).

In the process of endochondral ossification, the expression of TGase C was observed only at the hypertrophic chondrocytes of the central region of the callus(Fig. 5). Though it has been reported that the extracellular bone matrix proteins such as osteocalcin and osteonectin are the possible substrates of transglutaminase, the differences of those enzymes and substrates in expression period after fracture suggest the special aspect of bone formation. In our previous work, TGase was expressed in the resting and hypertrophic chondrocytes of the callus and the epiphyseal bone plate(Seong *et al.* 1993), where it was assumed that the crosslinking of a variety of nectins and other bone matrix proteins such as vitronectin, fibronectin, osteonectin and osteopontin by transglutamination reaction would provide a strong covalent network for structural stabilization enzymatically in addition to their fibrous noncovalent interacting capacity(Barsigian *et al.* 1991; Prince *et al.* 1991; Sane *et al.* 1988). But the present data showing the expression of TGase C later than the bone matrix proteins may imply that in the early bone formation proliferating activity and production of bone matrix proteins are very active, but in the late bone formation, the activation of TGase might crosslink the bone matrix proteins to be rearranged before the completion of bone formation.

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